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COCCIDIOIDOMYCOSIS: STUDIES OF CANINE VACCINATION AND THERAPY

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A number of evaluations of the efficacy of nonviable vaccines against experimental coccidioidomycosis have been made in laboratory animals. Negroni et al (1949) and Vogel et al (1954) used guinea pigs for their studies. Friedman and Smith (1956), Levine et al (1960, 1961), Converse et al (1962), and Kong et al (1963) used mice. Levine et al (1962) and Sinski et al (1963) used monkeys. All these investigators found that, although survival time of the immunized animals could be extended by the vaccines used, the majority of infected animals harbored viable *Coccidioides immitis* for long periods after challenge.

Campbell and Hill (1959) in their studies of therapy in experimental coccidioidomycosis of mice found that the presolubilized form of amphotericin B for intravenous use was more readily absorbed from the gastrointestinal tract than was the preparation made for oral use. The drug was well tolerated and demonstrated no apparent clinical side effects. These investigators were able to prolong survival in mice challenged with *C. immitis* by mixing amphotericin B in the drinking water.

The purpose of this study was to compare in the dog (a) the efficacy of a nonviable vaccine administered via the pulmonary route with that administered subcutaneously and (b) to determine the degree of protection afforded by the vaccinations in combination with amphotericin B therapy.

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Animals were maintained in compliance with the "Principles of Laboratory Animal Care" as promulgated by the National Society for Medical Research (1961, Bio-Medical Purview 1:14).

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METHODS

Organism.—The organism used in these experiments was *C. immitis*, strain Silveira, grown on modified Sabouraud (0.1% yeast extract) agar plates at 34 C until arthrospores were formed and harvested as a dry powder by vacuum apparatus after desiccation of the plates.

Vaccine.—Killed vaccines were prepared by exposure of the arthrospores to 0.5% aqueous formaldehyde at 25 C for 48 hours. The arthrospores were then washed and resuspended in normal saline at concentrations of 8 mg per ml (dry weight) for the subcutaneous and intratracheal vaccines and 40 mg per ml for the aerosol vaccine.

Vaccination.—Pulmonary vaccination was accomplished either by inhalation of the aerosolized vaccine or by intratracheal instillation of the spore suspension.

A plastic box with a volume of 2 cubic feet (equipped with an air filter to maintain normal air pressure within the box) was used as an aerosol chamber. Two 3-inch ports covered with slit rubber diaphragms permitted the insertion of the dogs' muzzles into the chamber for a 10-minute exposure to the aerosol. The nozzle of a no. 15 DeVilbiss nebulizer was inserted through a small hole in the

end of the box. Four ml of the vaccine (40 mg per ml) were aerosolized in the chamber over a period of 3 minutes.

Intratracheal vaccination was accomplished after tranquilizing the animals with Sparine (promazine hydrochloride, Wyeth Laboratories, Inc.). One hand of the assistant was placed over the mouth and muzzle of the supine animal, 1 ml of the vaccine (8 mg per ml) was injected intratracheally 3 cm below the larynx from a 2-ml hypodermic syringe fitted with a three-quarter-inch 20-gauge needle, the animal was immediately lifted with the head up, and the airway was occluded momentarily. Removal of the hand from the muzzle resulted in immediate inspiration of the vaccine suspension in the trachea.

Subcutaneous vaccination was made by injection of 1 ml of the vaccine (8 mg per ml) in the lateral thorax immediately posterior to the scapula.

All vaccinations were repeated after a 2-week period.

Respiratory challenge.—The challenge aerosol was generated in a 6200-liter test chamber (Wolfe, 1961) by compressed air. The force generated by rupture of the diaphragm covering the end of a metal tube containing viable, dry *C. immitis* arthrospores disseminated them throughout the chamber. The theoretical inhaled dose was calculated from the cloud concentration, the average volume of canine lungs, the canine respiration rate, and the length of exposure. Cloud concentration was determined by plate counts of the contents of filter paper samplers through which measured amounts of the aerosol had been drawn.

Therapy.—Oral therapy with presolubilized amphotericin B (Fungizone, E. R. Squibb & Co.) was administered either in the drinking water or mixed with the food in a split dose of 75 mg twice a day for 20 days. The total dose approximated 3 g.

Pathology.—The animals were sacrificed with veterinary Nembutal (Abbott Laboratories). Their gross pathology was recorded, and tissues from all viscera were fixed in 10% formalin, embedded in paraffin, serially sectioned, and examined histologically after staining with Giemsa, Gomori silver methenamine, and Ziehl-Neelsen acid-fast stains.

RESULTS

Three groups of mature, mixed breed dogs of both sexes, 7 to 10 kg in weight, vaccinated either subcutaneously, intratracheally, or via the respiratory route by inhalation, received a respiratory challenge of approximately 80,000 arthrospores 30 days after the second vaccination, as did another group of nonvaccinated dogs. Unchallenged treated dogs and vaccinated dogs (2 per group) were maintained as drug and vaccine controls.

Immediately after respiratory challenge half of the dogs in each of the 4 experimental groups were placed on an oral amphotericin B therapy regimen of 150 mg per day (dissolved in the drinking water) for 20 days. Eight weeks after challenge complete necropsies with histological studies of all internal organs were made on all animals.

Figures 1 through 8 illustrate the pathological changes noted in the animals in each of 2 experiments. Each photograph is representative of a large number of serial tissue sections from all animals in its respective group. In addition, figures 5 through 8 serve to visualize the terms negative, minimal, moderate, and severe pathological involvement, referred to in tables 1 and 2.

In the first experiment neither of the pulmonary routes of vaccination provided protection alone or in combination with amphotericin B, nor was protection provided by amphotericin B

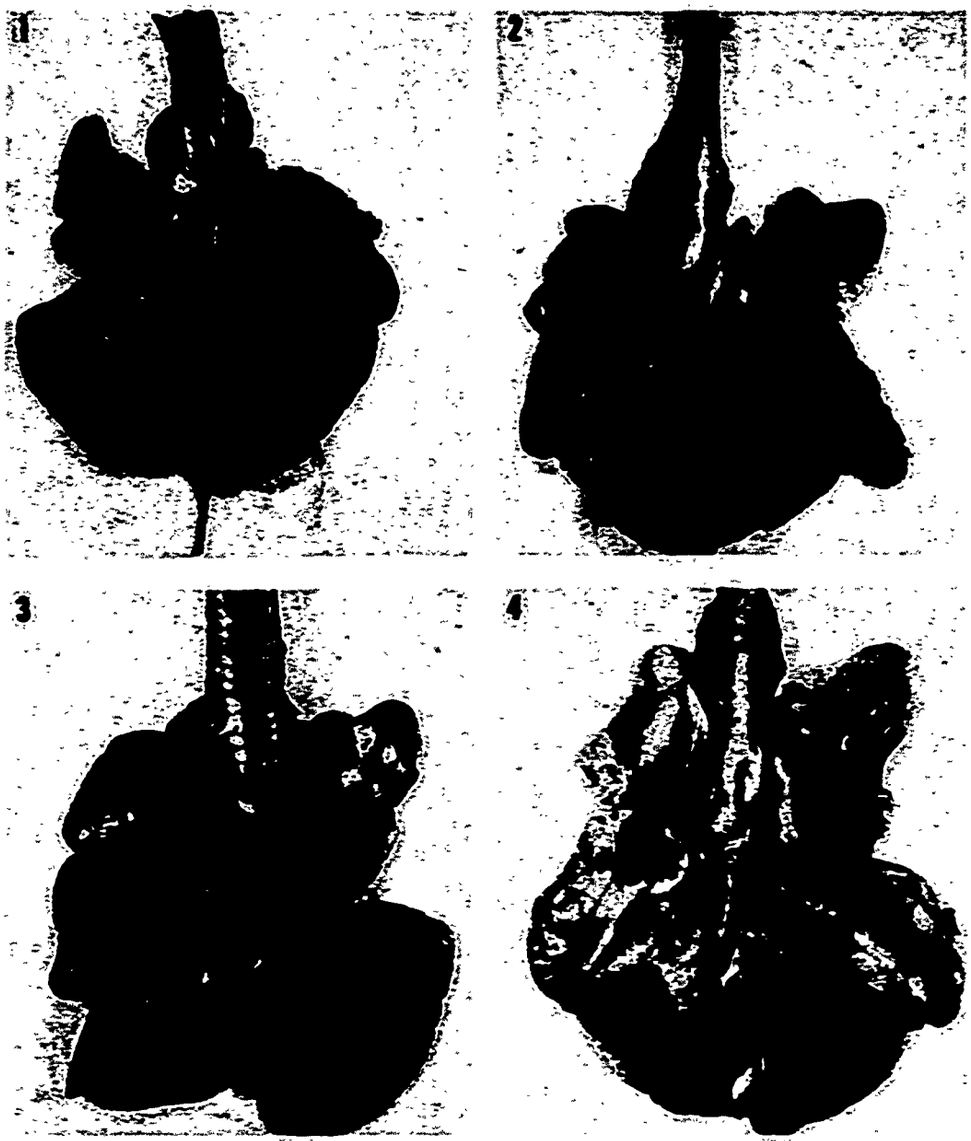
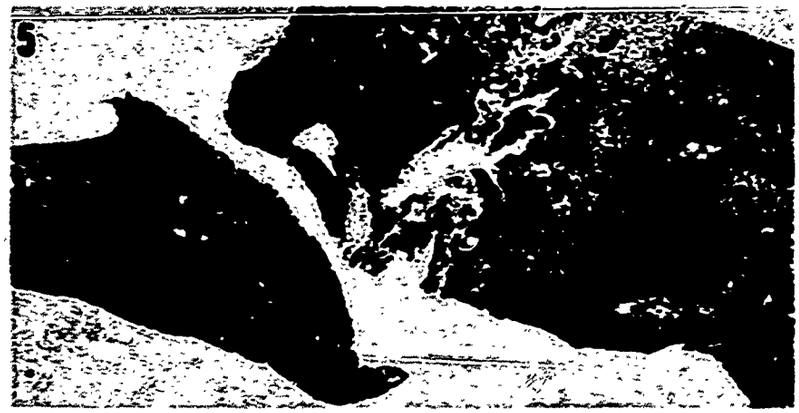


FIGURE 1.—Lungs from dog L44, subcutaneously vaccinated.

FIGURE 2.—Lungs from dog 311I, treated with oral amphotericin B. Both L44 and 311I received a respiratory challenge of 80,000 *Coccidioides immitis* arthrospores. Note bosselated appearance due to surface and subsurface coccidioidal lesions.

FIGURES 3 AND 4.—Lungs from dogs L38 and T80, respectively. These animals received both subcutaneous vaccine and therapy, the former a respiratory challenge of 80,000 and the latter 7000 arthrospores. Note the essentially normal appearance of these lungs as contrasted to those of figures 1 and 2.

alone (table 1). The animals in these groups and in the nonvaccinated control group developed moderate to severe pulmonary coccidioidomycosis, and in 1 instance, dog 74L, there was dissemination to the liver, spleen, and adrenal glands. On autopsy the surfaces of the lungs were bosselated in appearance (figures 1 and 2), and there were many scattered, palpable nodules throughout



FIGURES 5 AND 6.—Stained histological sections of the lungs of dogs L38 and T80, respectively (figures 3 and 4). L38 was microscopically diagnosed as negative to coccidioidomycosis, T80 as minimally involved. Three focalized granulomata may be seen in figure 6. 75X.

all lobes. On sectioning these nodules gave the appearance of caseous, necrotic granulomata (figure 7). Microscopic examination revealed many *C. immitis* spherules in all stages of development. Protection was noted only in the subcutaneously vaccinated and treated ani-

TABLE 1.—Protective effect of route of vaccination and amphotericin B on the development of pulmonary coccidioidomycosis in dogs

Vaccination route	Treated		Not treated	
	Animal	Results	Animal	Results
Aerosol	98L	++++	74L	++++
	9K2	+++	3M4	++++
	2M9	++	9K6	+
Subcutaneous	L38	0	3K7	+
	L73	0	L91	++++
	L32	0	2k0	+++
Intratracheal	4M8	+	M95	+++
	7M1	+	4L9	0
	99K	+++	31M	++++
Not vaccinated (controls)	9X2	+++	35X	++++
	31H	++++	L00	+
	M52	+++	41X	++++

0, negative; +, minimal; ++, moderate; +++, severe, no dissemination; ++++, severe, dissemination.

TABLE 2.—Protective effect of subcutaneous vaccination and amphotericin B on the development of pulmonary coccidioidomycosis in dogs

Vaccinated: treated		Not vaccinated: not treated	
Animal	Results	Animal	Results
T80	+		
73S	+		
51S	0		
T19	0		
X70	+		
67T	0	72I	++++
68R	+	7S1	+++
T41	0	R99	++++
68S	0	T67	++++

0, negative; +, minimal; ++, moderate; +++, severe, no dissemination; ++++, severe, dissemination.

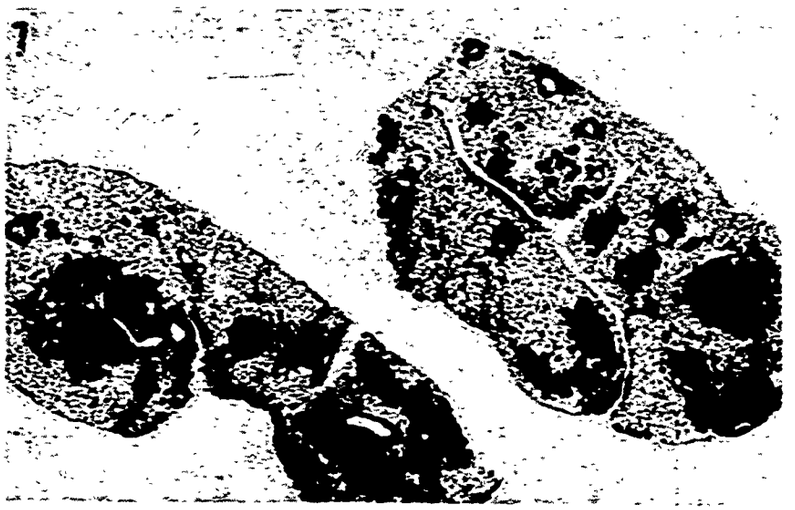


FIGURE 7.—Stained lung section from dog L44 (figure 1), diagnosed as moderately involved. 1.5X.
FIGURE 8.—Stained section from lung of dog R99, diagnosed as severely involved. This dog was a nonvaccinated, untreated control animal receiving a respiratory challenge dose of 7000 arthrospores. Note the numerous lesions in figures 7 and 8, showing consolidation and evidence of caseous necrosis. 1.5X.

nals (figures 3 and 5). Several dogs in the subcutaneously vaccinated group developed sterile abscesses at the vaccination site. Dog 6L9, presumably vaccinated intratracheally, developed a similar abscess to the right of the trachea following his first vaccination. This, essentially a subcutaneous vaccination, may explain the lack of histopathological changes in this animal.

The promising results obtained from the subcutaneously vaccinated and treated dogs led to a repetition of this portion of the work in a second experiment. All the conditions of the first experiment were repeated, except that 9 dogs were subcutaneously vaccinated instead of 3 as before. These 9, plus 4 additional unvaccinated, untreated dogs, were then challenged via the respiratory route with an average inhaled dose of 7000 arthrospores. Amphotericin B (150 mg per day for 20 days) was again dissolved in distilled water,

but for this experiment it was administered as a split dose given twice daily mixed with the animal's food. In both experiments close attention was given to assure the dog's full daily receipt of drug. No vomiting, diarrhea, or other untoward reactions were ever noted. Two unchallenged untreated dogs served as environmental room controls.

Ten weeks after challenge all animals were necropsied. The results of this experiment are shown in table 2. In striking contrast to the severe disease in the control animals (figure 8), only 4 of the 9 dogs receiving vaccination and therapy demonstrated any histopathological changes. These changes were very minimal (figure 6) and indicative of self contained disease.

The vaccine control dogs (vaccinated, untreated, unchallenged), the environmental controls (nonvaccinated, untreated, unchallenged), and the drug controls (nonvaccinated, treated, unchallenged) were without histological interest. Intensive examination of the kidney tissues of all dogs receiving therapy amounting to a total dose of more than 3 g failed to disclose renal changes due to amphotericin B.

DISCUSSION

Hitchner and Reising (1952) demonstrated the feasibility of imparting immunity by inhalation of aerosolized attenuated microorganisms. As pointed out by Aleksandrov and his associates (1958), one of the principal advantages of this method is the ease and rapidity of vaccinating large groups. Eigelsbach and co-workers (1961) found evidence that aerogenic vaccination with attenuated *Pasteurella tularensis* afforded perhaps greater immunity against tularemia than did the dermal route.

Sinski et al (1963) reported decreased mortality in monkeys immunized with killed, aerosolized *C. immitis* arthrospores, although all of the animals contracted pulmonary coccidioidomycosis when exposed to the live organism via the respiratory route. The results obtained in our study also indicated that the immune mechanism was not sufficiently stimulated by inhalation of killed arthrospores to prevent infection. It is postulated that the lung clearance mechanism disposed of the arthrospores as nonviable particulate matter in sufficient quantity to prevent an apparent response.

No explanation is offered for the sterile abscesses that occurred at the injection site in the subcutaneously vaccinated dogs. This reaction was subsequently greatly reduced, but not entirely eliminated, by dividing the dose and administering it in separate sites.

The nonvaccinated but treated dogs were surprising in their positive response to challenge. Previous amphotericin B therapy of mice in these laboratories (Converse et al, 1963) had substantiated the results of Campbell and Hill (1959), and it had been believed that the dogs would at most show only minimal response to challenge.

Cultural studies of the lung tissues in these experiments were not made, since previous experience in our laboratories had shown that, almost without exception, *C. immitis* can be cultured from the lungs of nonvaccinated challenged mice, monkeys, or dogs and that it is very difficult to culture *C. immitis* from vaccinated animals with minimal, focalized, self contained coccidioidomycosis, even when spherules can be seen in histological sections stained with specific fungus stains. In the latter instance the possibility exists that, even though the organism can be seen in the lesion, it may be nonviable.

Bell et al (1962) have reported in an addendum the possible association of human and canine renal tubular damage

with intravenous administration of amphotericin B. Sanford et al (1962) described a nephrocalcinosis and marked morphological changes in 3 renal biopsies of human patients, with distinct renal functional deficits being noted in all patients intravenously treated with amphotericin B. However, no histological evidence of renal damage attributable to the use of amphotericin B was seen in the 2 drug control dogs or in any of the animals receiving treatment. A slight increase in the blood urea nitrogen values of the drug-control dogs was noted during and shortly beyond the test period. However, these values remained well within normal limits. Although the blood serum levels of the drug were not determined in our dogs, the absence of renal damage is attributed to the small amounts absorbed into the blood stream from the digestive tract.

It has been shown in this paper, and by Castleberry et al (1962) and Sinski et al (1963), that dogs exposed to respiratory doses as low as 2000 to 10,000 *C. immitis* arthrospores can be expected to develop severe or disseminated (extrapulmonary) disease. The number of dogs used in this study is admittedly small. However, 8 of 12 dogs subcutaneously vaccinated and subsequently treated were negative to severe aerosol challenge with 7000 to 80,000 arthrospores. The remaining 4 responded very minimally.

It is evident that the incidence and severity of coccidioidomycosis in aerosol-challenged dogs can be safely and materially reduced by combining the physiological effects of a subcutaneously administered killed arthrospore vaccine with daily, orally administered, presolubilized amphotericin B. If a coroliary may be drawn between man and dogs, this may well be an excellent protection to workers in the field of coccidioidomycosis, i.e., routine subcutaneous injection of a vaccine of this type or of the type developed by Levine et al (1962), combined with low oral doses of amphotericin B at the time of known laboratory accidents.

SUMMARY

A 3-phase study of vaccination and antibiotic therapy in experimental pulmonary coccidioidomycosis of dogs was made to determine (a) the efficacy of various routes of inoculation of a formaldehyde-killed arthrospore vaccine, (b) the combined effects of vaccination and oral amphotericin B therapy administered immediately following respiratory exposure to *Coccidioides immitis*, and (c) renal damage or nephrotoxicity resulting from oral amphotericin B therapy. Neither of the pulmonary routes of vaccination (aerosol or intratracheal), either singly or in combination with oral amphotericin B therapy (150 mg per day for 20 days following challenge), provided protection against a subsequent respiratory challenge of approximately 80,000 *C. immitis* arthrospores. Neither subcutaneous vaccination nor therapy alone provided protection. However, 8 of 12 dogs receiving both subcutaneous vaccination and therapy completely resisted the respiratory challenge. The remaining 4 exhibited very minimal, self contained disease. Histopathological examination revealed no renal damage or nephrotoxicity in any of the dogs receiving amphotericin B in a total dose of more than 3 g, and their blood urea nitrogen levels remained within normal limits.

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